APPENDIX A

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Laurence CHRISTA *et al.*	Group Art Unit: 1646
Serial No.: 10/561,034	Examiner: Howard, Zachary C.
Filed: December 16, 2005	Atty. Dkt. No.: CHEP:015US/10513205
For: HIP/PAP POLYPEPTIDE COMPOSITION FOR USE IN LIVER REGENERATION AND FOR THE PREVENTION OF LIVER FAILURE	Confirmation No.: 9442

CERTIFICATE OF ELECTRONIC TRANSMISSION
37 C.F.R. § 1.8

I hereby certify that this correspondence is being electronically filed with the United States Patent and Trademark Office via EFS-Web on the date below:

Oct. 19, 2010
Date

Mark B. Wilson

DECLARATION OF Dr. JAMILA FAIVRE UNDER 37 C.F.R. §1.132

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

I, Jamila Faivre (MD, PhD) do declare that:

1. I am Associate Professor of Cell Biology employed by the University Paris 11, Orsay, France and Paul-Brousse Hospital, Villejuif, France. I am senior scientist and research group leader in the Research Unit 785 (Paul-Brousse Hospital, Villejuif, France) of the Institut National de la Santé et de la Recherche Médicale (INSERM). I have had these positions since 2003. I received a medical degree from University Paris 13 in 1990, a Doctor of Philosophy degree in Cell Biology and Biochemistry from University UPMC

Paris 6 in 2004, and a research habilitation from University Paris 11 Orsay in 2009. I have about 15 years of working experience in clinical and health science research. My research activities have focused on liver regeneration and carcinogenesis since 2006. I have research experience in the field of Hepatology, Cancerology, Molecular and Cell Biology. A copy of my curriculum vitae and my list of publications on this topic is attached as Exhibit 1.

2. I am providing this declaration to describe certain studies performed in the research unit INSERM U785 at the Hepatobiliary Centre of the Paul-Brousse Hospital, Villejuif, under my direction. These studies were performed to compare the anti-apoptotic activity of a polypeptide having amino acids 36-175 of the hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein (HIP/PAP), called 36–175 HIP/PAP, to the biological activity of a polypeptide having amino acids 27–175 of HIP/PAP, called 27–175 HIP/PAP. This declaration describes the experiments performed and the results obtained. Additional details regarding the experiments can be found in the attached report, which is Exhibit 2, and the references cited therein.

3. To study the anti-apoptotic activity of the HIP/PAP polypeptides (also called ALF-5755 in Exhibit 2), a caspase 3 inhibition test was performed. This study evaluated the ability of the HIP/PAP polypeptides to inhibit apoptosis in rat hepatocytes in primary culture after the hepatocytes had been stimulated to undergo apoptosis.

4. The 36–175 HIP/PAP and 27–175 HIP/PAP were prepared and provided by PX'Pharma, Grenoble, France. The 36–175 HIP/PAP sample (also called PX7-F1-ALF-5755-291007 or FL-ALF-5755 in Exhibit 2) contained approximately 94% 36–175 HIP/PAP. The 27–175 HIP/PAP sample (also called I13-D150 or sALF-5755 in Exhibit 2) contained approximately 97% 27–175 HIP/PAP.

5. Fresh rat hepatocytes were cultured following standard operating procedures. Briefly, primary hepatocytes were prepared from adult female Wistar rats (5 to 8 weeks old, Charles River Laboratories Inc. MA, USA) according to a published experimental procedure. A standard 2-step liver perfusion was performed (using 500 mL of 40°C pre-warmed Hepes pH 7.65 + EGTA 0.09% followed by 300 mL Hepes / collagenase 0.25 mg/mL / $CaCl_2$ 0.75 mg/mL) to avoid massive hepatocyte death during trituration. Tissue dissociation was performed in L15 medium complemented with antibiotics (penicillin 100 units/mL, streptomycin 100 µg/mL), fungizone (250 ng/mL), and BSA (1 mg/mL). After filtration on a 70 µM nylon membrane, cells were centrifuged at 600 rpm for 2 minutes to specifically pull down hepatocytes. Cells were rinsed twice in complemented L15 medium. Viable cells were counted by Trypan blue exclusion and plated in 10% FCS complemented William's medium + antibiotics (penicillin 100 units/mL, streptomycin 100 µg/mL), fungizone (250 ng/mL), and BSA (1 mg/mL). 30,000 cells per well were seeded in 96 well plates. Once attached (3 to 4 hours post-plating) cells were switched to FCS-free complemented William's medium, which was replaced every day.

6. Efforts were made to achieve the highest possible post-trituration viability. Viability below 70% disqualified the experiment. For the caspase 3 inhibition experiments, the number of living cells per well in the basal conditions (no treatment) at the time of apoptotic induction should be reasonably high to allow a large panel of rescuing response when treated. Caspase 3 induction should reach a magnitude of about 4 to 10 in the control condition (apoptotic condition) to maintain a certain number of living cells and allow cell death rescue. Any experiment that did not meet these criteria was disqualified.

7. Apoptosis was induced in cultured hepatocytes by subjecting them to a mixture of Tumor Necrosis Factor α (TNFα) and ActinomycinD at 10/50 or 20/50 ng/mL, respectively.

Caspase 3 cleavage (caspase 3 activity) was measured with a Promega Casp3-7 kit; experiments were done as recommended by the provider. Each condition was tested in octoplicates per experiment. The effect of increasing doses of both forms of HIP/PAP ranging from 20 to 4000 ng/mL was investigated.

8. Raw data were expressed as arbitrary units of chemoluminescence. In each experiment, data were compared to a positive control (PC: apoptotic stimulus) and a negative control (NC: no apoptotic stimulus = basal condition). Statistical evaluations of significance within experiments were applied to raw data as all measurements were done simultaneously. Because the quantity of chemoluminescence varied from one experiment (or plate) to another, inter-experiment (or plate) analysis was performed. To perform inter-experiment (or plate) analyses, we normalized all data to their respective negative and positive controls. For each chemoluminescence value X, we calculated the ratio RX = (X – mean of NC) / (mean of PC – mean of NC). By definition, the mean of NC is 0 when the mean of PC is 1. The ratio RX is the percentage of caspase 3 activity when the animal is treated by the HIP/PAP forms. A dose-response was performed for each condition, and non-parametric Kruscall-Wallis tests followed by Dunns post-hoc tests were applied to reveal HIP/PAP effects according to batches. IC50 values were calculated in every condition. Two-way ANOVA was performed to compare batches, and post-hoc Bonferroni corrections were applied to reveal effects of HIP/PAP concentration. All indices of fidelity were calculated for an interval of confidence of 95%. Analyses were performed with Graph-Pad software.

9. To assess biological activity of the HIP/PAP polypeptides, two experiments (080715-APOP127, 080721-APOP129) were performed with apoptosis induction mediated by TNFα/ActD mixtures at 10/50 and 20/50 ng/mL. For each condition, 8 replicates were

performed. The HIP/PAP anti-apoptotic activity found with the two mixtures were comparable for both HIP/PAP forms; therefore, all data were pooled for analyses.

10. The calculated IC_{50} values for 36–175 HIP/PAP and 27–175 HIP/PAP were 264 and 238 ng/mL respectively, with overlapping intervals of confidence (175–399 and 139–410). One-way ANOVA analysis showed a statistically significant effect of HIP/PAP from 100 ng/mL upward for the 2 batches with *P* values below 0.0001 from 250ng/mL for sALF-5755 and from 100ng/mL for FL-ALF-5755. Two-way ANOVA analysis showed no difference in activity for the two forms of HIP/PAP with $F_{(558,1)} = 0.003$ ($P = 0.95$) and no interaction with $F_{(558,8)} = 0.68$ ($P = 0.71$). As illustrated in *Graph 1* of Exhibit 2, Bonferroni post-hoc tests showed no difference for each ALF-5755 form, at all concentrations (all the *P* values were above 0.05).

11. These results show that the anti-apoptotic activity of 36–175 HIP/PAP was as high as that of 27–175 HIP/PAP in rat hepatocyte primary cultures. The IC_{50} values of the two proteins were of 264 and 238 ng/mL, respectively, and had widely overlapping intervals of confidence (175-399 and 139-410 ng/mL respectively). In other words, the IC_{50} values of the two HIP/PAP polypeptides were not significantly different. In conclusion, 36–175 HIP/PAP, a polypeptide having 93.9% identity to amino acids 27–175 of HIP/PAP, exhibited the same anti-apoptotic activity as a polypeptide having 100% identity to amino acids 27–175 of HIP/PAP.

12. I declare that all statements made herein of my own knowledge are true, and that all statements of my own belief are believed to be true, and further that these statements were made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under § 1001 of title 18 of the United States Code.

Date 09/22/2010

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EXHIBIT 1

Curriculum Vitae
Jamila FAIVRE

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Born: July 20, 1960 (France). Nationality: French. Married, 3 children.
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Academic Degrees
1990 Doctor in Medicine (MD), Medical Thesis.
1992 Certificates of Virology and Genetic Engineering, Pasteur Institute, Paris.
1993 Qualified for Medical Microbiology. University Paris 6 - Faculty of Medicine Broussais-Hôtel-Dieu.
1995 Master's Degree, Pierre and Marie Curie Paris University.
2004 Doctor of Pierre and Marie Curie Paris University (PhD).
2009 Habilitation to be Director of Research, University Paris 11.

1998-2004 Assistant Professor in Cell Biology and Hepatology.

Current Position
Associate Professor of Cell Biology since September 2005.
Research group leader in the Research Unit 785 « Physiopathology and Treatment of Fulminant Hepatitis and Liver Cancer »

Publications:
Faivre J, Franck-Vaillant M, Poulhe R, Mouly H, Brechot C, Jessus C and Sobczak-Thepot J. Centrosomes overduplication, increased ploidy and transformation in cells expressing endoplasmic reticulum-associated cyclin A2. **Oncogene**. 2002; 21 (10): 1493-1500

Faivre J, Clerc J, Gerolami R, Herve J, Longuet M, Liu B, Roux J, Moal F, Perricaudet M, Brechot C. Long-term radioiodine retention and regression of liver cancer after Natrium Iodide Symporter gene transfer in Wistar rats. **Cancer Research.** 2004; 64, 8045-8051

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Liu B, Herve J, Bioulac-Sage P, Valogne Y, Roux J, Yilmaz F, Boisgard R, Guettier C, Cales P, Tavitian B, Samuel D, Clerc J, Brechot C, **Faivre J**. Sodium iodide symporter is expressed at the preneoplastic stages of liver carcinogenesis and in human cholangiocarcinoma. **Gastroenterology.** 2007 ;132 (4):1495-1503

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N Moniaux, H Song, M Darnaud, K Garbin, M Gigou, C Mitchell, D Samuel, L Jamot, P Amouyal, G Amouyal, C Bréchot, and **J Faivre**. HIP/PAP cures Fas-induced Acute Liver Failure in mice by attenuating free-radical damage in injured livers. 2010. Hepatology In revision

EXHIBIT 2

- REPORT -

Title:
Comparison of short *versus* full-length ALF-5755 activity in primary culture of rat hepatocytes

STUDY MANAGEMENT

Testing Laboratory:	**Inserm U785** Director: Prof. **Didier Samuel, MD, PhD** Pathogenesis and Treatment of Fulminant Hepatitis and Liver Cancer Hepatobiliary Center Paul Brousse Hospital 12 av. Paul Vaillant Couturier 94800 Villejuif France 01 45 59 60 74
Study Director:	**Jamila Faivre, MD, PhD (Inserm U785)**
Scientific Writer	**Laure Jamot, Ph.D. (Alfact Innovation)**
Sponsor:	**Alfact Innovation** **President: Paul Amouyal, MD** 320, rue Saint Honoré 75001 Paris Tel : 01 53 40 60 51 Fax : 01 53 40 60 52 Email: amouyal.paul@wanadoo.fr

STUDY SITE

Study was performed at:
Inserm U785
Pathogenesis and Treatment
of Fulminant Hepatitis and Liver Cancer
Hepatobiliary Center
Paul Brousse Hospital
12, avenue Paul Vaillant Couturier
94800 Villejuif
France

Study Operators:

Study Operator:	Laure Jamot (Alfact Innovation)

I. AIM AND DESIGN OF THE STUDY

The aim of this study was to investigate the biological activity of sALF-5755 (36-175 HIP/PAP form) compared to that of full-length ALF-5755 (Met-27-175 HIP/PAP form).

For this purpose we compared the biological activity of the following two products:
- a standard batch of ALF-5755 containing more than 97% of Met-27-175 HIP/PAP form,
- a batch comprising sALF-5755 containing more than 93% of 36-175 HIP/PAP obtained by treating the standard batch with trypsin.

The biological test for studying HIP/PAP activity was a caspase 3 cleavage inhibition test in rat hepatocytes in primary culture under the stimulation of an apoptotic stress.

II. MATERIALS AND METHODS

1) Test items

a. ALF-5755 batches

- For FL-ALF-5755: batch PX7-F1-ALF-5755-291007 was manufactured and provided by PX'Pharma. This batch was received at Inserm U785 in November 2007 at a concentration of 344 ng/mL; upon arrival, it was aliquoted and stored at -80°C.
- For sALF-5755: batch I13-D150 was prepared in PX'Pharma facilities by processing 6 mL of batch PX7-F1-ALF-5755-291007 onto a trypsin column. Three vials of 200 µL (concentration:130 ng/mL) were sent to Inserm U785 and immediately stored at -80°C.

The formulation buffer was also provided by PX'Pharma.

The following table (Table 1) recapitulates the composition of the 2 batches:

Table 1

Batch reference	HIP/PAP form	
	36-175 HIP/PAP	Met-27-175 HIP/PAP
PX7-F1-ALF-5755-291007	3%	97%
I13-D150	93.75%	6.25%

b. ALF-5755 dosage preparation

ALF-5755 was applied as a solution in its formulation buffer added to the culture medium. The effect of increasing doses of ALF-5755 ranging from 20 to 4000 ng/mL was investigated. The dosage forms were prepared extemporaneously. ALF-5755 was mixed with the required quantity of medium in order to achieve the maximal tested concentration of 4000ng/mL. Lower concentrations were obtained by diluting the ALF-5755 4000ng/mL solution with appropriate volumes of culture medium.

2) Cell culture

Fresh rat hepatocytes were cultured following standard operating procedures (IVTA-SOP1). Briefly, primary hepatocytes were prepared from adult female Wistar rats (5 to 8 weeks old,

Charles River Laboratories Inc. MA, USA) according to a published experimental procedure[7,8]. A standard 2-step liver perfusion was performed (500 mL of 40°C pre-warmed Hepes pH 7,65 + EGTA 0.09% followed by 300 mL Hepes / collagenase 0.25 mg/mL / $CaCl_2$ 0.75 mg/mL) to avoid massive hepatocyte death during trituration. Tissue dissociation was performed in L15 medium complemented with antibiotics (penicillin 100 units/mL, streptomycin 100 µg/mL), fungizone (250 ng/mL), and BSA (1 mg/mL). After filtration on 70 µM nylon membrane, cells were centrifuged at 600 rpm for 2 minutes to specifically pull down hepatocytes. Cells were rinsed twice in complemented L15 medium. Viable cells were counted by Trypan blue exclusion and platted in 10% FCS complemented Williams's medium + antibiotics (penicillin 100 units/mL, streptomycin 100 µg/mL), fungizone (250 ng/mL), and BSA (1 mg/mL). 30,000 cells per well in 96 well plates were seeded. Once attached (3 to 4 hours post plating) cells were switched to FCS-free complemented William's medium, which was replaced every day.

Post-trituration viability must be as high as possible since it is a marker for cell integrity, which will impact on the cell response to apoptotic stimuli and rescuing capacities of the tested drugs. Viability below 70% would disqualify the experiments. In addition, for caspase3 inhibition experiments, the number of living cells per well in the basal conditions (no treatment) at the time of apoptotic induction should be reasonably high to allow a large panel of rescuing response when treated. Caspase 3 induction should reach a magnitude of about 4 to 10 in the control condition (apoptotic condition) to maintain a certain number of living cells and allow cell death rescue. Not meeting these criteria would disqualify the experiments.

3) Reagents

The following list displays all the reagents used during the course of the experiments:

Reagent	Supplier	Reference
HEPES	Sigma	H6147
Sterile water	B. Braun	0066571E
NaCl	Sigma	S5886
Sodium phosphate	Sigma	S5136
Potassium chloride	Sigma	P5405
EGTA	Sigma	E3889
CaCl2	Sigma	C7902
Collagenase	Sigma	C5138
William's medium + glutamax	Gibco Invitrogen	32551-020
BSA	PAA	K11-013
Penicillin/ Streptomycin	Gibco Invitrogen	15140-122
FCS	PAA	A15-101
Fungizon	Gibco invitrogen	15290-026
Dexamethasone	Sigma Aldrich	D4902
Insulin	Sigma Aldrich	I0516
96 well-plates	NUNC	136101
TNFα	Sigma	T7539
Actinomycine D	Sigma	A1410
Caspase inhibitor, Ac-DEVD-CHO	Promega	G5961
Caspase Glo 3/7	Promega	G8091

4) Induction of apoptosis and read-out

Apoptosis was induced in cultured hepatocytes by subjecting them to two combinations of Tumor Necrosis Factor α (TNFα) and ActinomycinD at 10/50 or 20/50 ng/mL, respectively. This was based on previous work[9] and data (see reports IVTA-Rat1 to 3). Caspase 3 cleavage (caspase 3 activity) was measured with Promega Casp3-7 kit. Experiments were done as recommended by the provider. Each condition was tested in octoplicates per experiment. The effect of increasing doses of both forms of ALF-5755 ranging from 20 to 4000 ng/mL was investigated.

5) Statistical analyses

Raw data are expressed in arbitrary units of chemoluminescence. In each experiment, data are compared to a positive control (PC: apoptotic stimulus) and a negative control (NC: no apoptotic stimulus = basal condition). Statistical evaluations of significance within experiments were applied to raw data as all measurements were done simultaneously.

However, from one experiment (or plate) to another, the quantity of chemoluminescence varies. Therefore, to perform inter-experiment (or plate) analyses, we normalized all the data to their respective negative and positive controls by calculating the following ratio for each chemoluminescence value X:

$R_X = (X - \text{mean of NC}) / (\text{mean of PC} - \text{mean of NC})$.

By definition the mean of NC will be 0 when the mean of PC will be 1. This ratio reflects the percentage of caspase 3 activity when treated by ALF-5755.

A dose response being performed for each condition, non-parametric Kruscall-Wallis tests followed by Dunns post-hoc tests were applied to reveal ALF-5755 effects according to batches. IC_{50} values were calculated in every condition. Two-ways ANOVA were performed to compare batches and post-hoc Bonferroni were applied to reveal effects of ALF-5755 concentration.

All indexes of fidelity were calculated for an interval of confidence of 95%. Analyses were performed with Graph-Pad software.

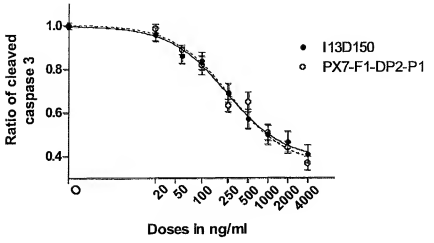
III. RESULTS

1) Assessment of sALF-5755 vs FLALF-5755 biological activity

Two experiments (080715-APOP127, 080721-APOP129) were performed with apoptosis induction mediated through TNFα/ActD combination at 10/50 and 20/50 ng/mL. For each condition 8 replicates were performed. ALF-5755 activity was comparable in the two conditions of TNFα for both ALF-5755 forms, therefore the corresponding data were pooled for analyses.

The calculated IC_{50} values for PX7-F1-ALF-5755-291007 and I30-D150 were 264, and 238 ng/mL, respectively, with overlapping intervals of confidence (175-399 and 139-410, respectively). One-way ANOVAs showed statistical significant effects of ALF-5755 from 100 ng/mL onward, for the 2 batches with P values below 0.0001 from 250ng/mL for sALF-5755 and from 100ng/mL for FL-ALF-5755. Two ways ANOVA showed no difference in activity for the two forms of ALF-5755 with $F_{(558,1)} = 0.003$, $P = 0.95$ and no interaction with $F_{(558,8)} = 0.68$, $P = 0.71$. However there was a statistically significant effect of ALF-5755 with $F_{(558,8)} = 82.3$, $P < 0.0001$. As will be seen in *Graph 1*, Bonferroni post-hoc tests showed no difference for each ALF-5755 form, at all concentrations with all P values above 0.05.

Figure 1: ALF-5755 batch PX7-F1-DP2-P1 and I30-D150 display the same anti-apoptotic activity



IV. CONCLUSION

These results show that the activity of 36-175 HIP/PAP (sALF-5755) was as high as that of the Met-27-175 HIP/PAP (full-length ALF-5755) in rat hepatocyte primary cultures. The IC_{50} values were practically the same for the two proteins (average values of 264 and 238 ng/mL, respectively, and widely overlapping intervals of confidence of 175-399 and 139-410 ng/mL respectively).

V. REFERENCES

Publications:

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